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The influence of different sera on the in vitro immobilisation of Percoll purified *Treponema pallidum*, Nichols strain

H J H Engelkens, M Kant, P C Onvlee, E Stolz, J J van der Sluis

Abstract

***Objectives*—Investigation of sera, especially rabbit serum, in preventing in vitro immobilisation of Percoll purified *T. pallidum*.**

***Materials and methods*—The immobilisation of Percoll purified *T. pallidum* (Nichols) was studied after pre-incubations with basal reduced medium (BRM), heat-inactivated serum of seven different species of animals, heat-inactivated normal human serum (NHS) and rabbit sera containing a different level of antitreponemal antibodies. Also increasing percentages of heat-inactivated normal rabbit serum (NRS) were studied.**

***Results*—The rapid immobilisation of purified treponemes by NHS is delayed by pre-incubation with NRS in a dose-dependent manner. The treponemes from 5-day infections were immobilised significantly more slowly than treponemes from 7- and 8-day infections. Compared with NRS, pre-incubations with a high-titred, low-titred and "autologous" serum resulted in significantly more rapid immobilisation of the treponemes. With most other animal sera resistance to immobilisation was slight compared with that produced by NRS. Immunofluorescent studies revealed that the treponemes were covered with a layer of the human third complement factor (C3b), within an hour of incubation. With two sequential pre-incubations, a delay of the immobilisation was only noted in those test mixtures in which NRS had been present in both pre-incubations.**

***Conclusion*—Rabbit serum delays the rapid in vitro immobilisation of Percoll purified treponemes by normal human serum. There was no evidence that this was caused by preventing access of antibodies (in vivo as well as in vitro) to, or preventing the activation of complement on, the treponemal surface. The evidence points to a mechanism in the fluid phase, suggesting participation of a third factor in the immobilisation process, for instance an enzyme, which can be partially inhibited by rabbit serum component(s).**

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Introduction

In vitro immobilisation of treponemes by antibodies and complement takes a long time: complete killing of treponemes by high-titred immune serum requires 16 h.[1] Treponemes may escape rapid destruction by the presence of a protective cover around the microorganisms. This cover may consist of mucopolysaccharides[2 3] or several serum proteins[4] and may prevent access of antibodies to the antigens located on the treponemal outer membrane. A paucity of antigens on the treponemal surface, as was recently demonstrated[1 5] may add to the ability of the treponemes to survive.

Treponemes for laboratory use are usually extracted from rabbit testes. As a consequence the treponeme suspensions are contaminated by rabbit components and possibly by substances produced during the infection. With Percoll density gradient centrifugation it is possible to obtain suspensions of motile and virulent treponemes relatively free of host proteins.[6 7] It was observed that Percoll purified treponemes were immobilised more quickly than unpurified treponemes in the presence of antibodies and complement.[8] After the addition of testicular extracts, purified treponemes became more resistant to immobilisation. Addition of testicular extracts originating from infected or uninfected rabbits made no difference, so that a possible role of substances produced under the influence of the infection was ruled out. However, it was not possible to determine whether this delay in immobilisation was due to an anti-complement activity of testicular extracts, or to a re-establishment of a protective cover around the treponemes.[8]

The ability of the treponemes to resist rapid immobilisation in the presence of rabbit testicular extracts on the one hand,[8] the presence of serum proteins in these extracts[9] and the close association of some of these proteins with the treponemal surface[4] on the other, prompted us to study a possible inhibitory effect of sera, particularly rabbit serum, on the immobilisation of purified *T. pallidum*, Nichols strain.

Material and methods

Propagation and extraction of T. pallidum. *T. pallidum* (Nichols) was maintained by intratesticular transfer in male New Zealand White rabbits.[9] At the time of inoculation, all animals had a negative Venereal Disease Research Laboratory (VDRL) test, *Treponema pallidum* Haemagglutination Assay (TPHA) and Fluorescent Treponemal Antibody-Absorbed (FTA-Abs) test. Treponemes were harvested 7

or 8 days after inoculation, unless otherwise stated. Extraction, Percoll purification and enumeration of *T. pallidum* were performed.[8]

Human serum pool. A pool of normal human serum (NHS) served as a complement source throughout this study. This pool was prepared from blood obtained from 150 donors with negative TPHA test results.

Rabbit serum pool. A pool of rabbit serum (NRS) was derived from 15 rabbits with negative VDRL, TPHA and FTA-Abs test results.

Both pools were stored in small aliquots at −70°C. Samples used in the experiments were thawed only once. Heat-inactivated samples were also used for the pre-incubation step of the treponeme suspensions.

Other animal sera. A serum sample with a TPHA titre of 1:10240, a VDRL titre of 1:8 and a 3+ FTA-Abs test result was obtained from a rabbit previously infected with *Treponema pallidum* (high-titred serum); a serum sample with a TPHA titre of 1:80 and negative VDRL and FTA-Abs results was obtained from a rabbit suffering from a *Treponema cuniculi* infection (low-titred serum). "Autologous" serum samples were also taken from the infected rabbits, on the day they were killed for extraction of the treponemes.

The following sera were also tested; sera from a pig (cross-breeding of Danish country pig and Yorkshire pig), a Syrian golden hamster, a goat (outbred goat), a mouse (C 57 black mouse), a dog (Beagle), a rat (Wistar) and a guinea-pig (Dunkin-Hartley). To exclude possible variation between animals of the same species, blood was taken from five representatives of four species of animals (pig, rabbit, dog and rat).

Heat inactivation of sera. The total complement activity of the sera, used for pre-incubation was destroyed by heating samples at 56°C for 30 minutes.

Estimation of complement activity. Optimally sensitised sheep erythrocytes were prepared. Before use in the immobilisation experiments, mixtures of NHS and NRS were tested for their haemolytic capacity in a set-up analogous to the CH_{50} method, using BRM as diluent.[8] The ratio of NRS to NHS in these mixtures was 2:2·5, being twice the ratio that was used in the immobilisation experiments (see below). The haemolytic capacity of these mixtures was compared with similar mixtures of BRM and NHS. The presence of residual haemolytic complement capacity in 22 h samples used in the immobilisation experiments was again tested by their ability to lyse the sensitised sheep erythrocytes.[10]

Immobilisation of treponemes in the presence of pre-incubation serum. Percoll treponemes were used in a final density of 2×10^7 treponemes per ml. A sufficient number of treponemes were pre-incubated with basal reduced medium (BRM),[11] dithiothreitol being omitted, or with the heat-inactivated human or various animal sera (final content 10%, unless otherwise stated) for 15 minutes. Subsequently NHS was added to a final content of 25% (v/v). Aliquots of 0·5 ml of these mixtures were placed in small tubes, which were loosely plugged with cottonwool and incubated in a reduced oxygen atmosphere (4%) at 34°C.[12] The percentage of mobile treponemes was determined in wet mounts after 0, 1, 2, 3·5 and 5·5 hours by observing at least 100 treponemes in randomly selected microscopic darkfields.

Immobilisation of treponemes after removal of pre-incubation serum. Two sequential pre-incubations of 15 min each of the Percoll purified treponemes were performed. After pre-incubation with NRS or BRM, the suspensions were centrifuged at 12 000 × *g* at 4°C for 10 min. The supernatant was removed. A part of the treponemes, which had been pre-incubated with NRS were further pre-incubated with NRS, the other part with BRM. Furthermore, treponemes were sequentially pre-incubated in BRM. After adding NHS as the complement source, the mixtures were processed as described above.

Fluorescence. After incubation periods of one and two hours in the immobilisation experiments, 10 ml phosphate-buffered saline (PBS) was added to 0·5 ml of the test mixtures. After centrifugation at 12 000 × *g* at 4°C for 10 minutes, the supernatant was removed and the pellet was suspended in 1 ml BRM. This suspension was pipetted into a 35 mm plastic Petri dish (Costar) equipped with a clean cover glass. After centrifugation at 800 × *g* for 5 minutes, the cover glasses were rinsed in BRM and overlaid with two drops of the FITC labelled IgG-fraction of a goat anti-human C3b antiserum (working dilution 50×) (Centraal Laboratorium van de Bloedtransfusiedienst, Amsterdam) and incubated at room temperature in a clean Petri dish for 30 min. After rinsing in BRM the cover glasses were placed upside down on microscopic slides and sealed with nail polish. They were read immediately. The optical system has been described.[13]

Statistical analysis. Wilcoxon's tests for paired and unpaired observations was used in the evaluation of results.

Results

Effect of NRS and NHS on immobilisation. Pre-incubation of the Percoll purified 7- and 8-days treponemes with BRM and subsequent addition of NHS as complement source resulted in rapid immobilisation. As shown in fig 1, almost all treponemes had lost their mobility after two hours. After pre-incubation with heat-inactivated NRS followed by addition of NHS, two-thirds of the treponemes retained their mobility after 2 hours, and after 5·5 hours over one-half of the treponemes showed good mobility in the presence of complement. In contrast, after pre-incubation with heat-inactivated NHS, followed by incubation with NHS, only 1·5% of the treponemes retained their mobility after 2 h (fig 1). Longer pre-incubation periods of up to three hours with heat-inactivated NHS did not change this result. The haemolytic capacity of the complement source was not impaired by addition of heat-inactivated rabbit serum: the presence of double the amount used in the immobilisation experiments did not change the CH_{50} level of

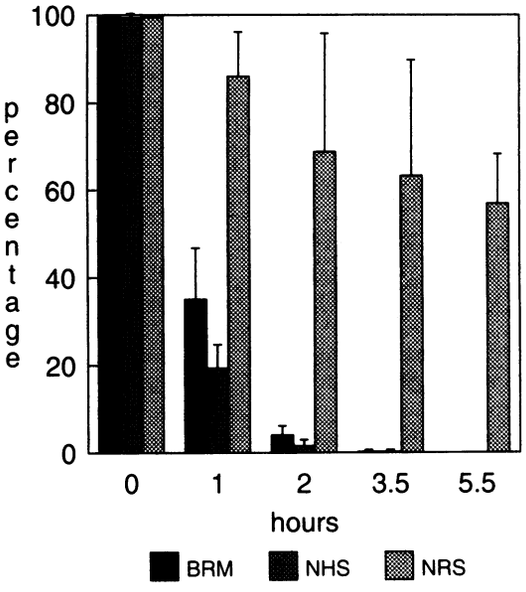

Figure 1 Immobilisation of Percoll purified treponemes; characteristics of preincubations with BRM, and inactivated normal human serum (NHS) versus inactivated normal rabbit serum (NRS). Normal human serum was used as a complement source throughout these experiments. Results are expressed in percentage of mobile treponemes. Means and standard deviations of the results of experiments with six treponemal suspensions originating from six different rabbits are shown.

the NHS pool in the lysis of optimally sensitised sheep erythrocytes (not shown).

Influence of the dose of NRS on immobilisation. Figure 2 shows the mean percentages of mobile treponemes at the various times after the treponemes had been pre-incubated with different amounts of NRS. Again, the treponemes pre-incubated in the absence of NRS had lost their mobility almost completely after 2 h. At this time the mean percentages of mobile treponemes in the NRS-containing reaction mixtures varied from 17% in mixtures containing 0·3% (v/v) NRS to 97% in mixtures containing 20% (v/v) NRS.

Influence of other animal sera on immobilisation. Pre-incubations of purified treponemes with the heat-inactivated sera from single representatives of seven different animal species all resulted in a delay of the immobilisation as compared with pre-incubations with heat-inactivated NHS. The effects of the hamster, goat, mouse, rat and guinea pig sera were slight. The dog serum had a stronger effect. The strongest effect was displayed by the pig serum (table 1). Results observed in experiments with sera from groups of five rabbits, five pigs, five dogs and five rats were the following. All five rabbit sera gave a strong inhibition of the rapid immobilisation, while the inhibition provided by the five rat sera was weak. The dog sera demonstrated an intermediate result. However, the pre-incubations with the sera of a group of five pigs gave varying results: the sera from two pigs provided strong resistance to immobilisation of the treponemes and with the three remaining sera the inhibition of immobilisation varied from moderate to very weak. All test mixtures in this set of experiments contained residual haemolytic complement activity. After 22 h of incubation the test mixtures were still able to haemolyse sensitised sheep erythrocytes.

Mobility of the treponemes and the presence of C3b on their surface. Immunofluorescent studies revealed that all treponemes in the experiments with the sera from the groups of animals were covered with a layer of the human third complement factor (C3b) within an hour of incubation. No relation was found between the rate of immobilisation and the presence of C3b on their surface after one or two hours.

Influence of duration of infection on immobilisation. As is shown in fig 3, Percoll-purified treponemes harvested after 5 days of infection and pre-incubated in BRM were immobilised significantly less rapidly than similarly treated treponemes, harvested after 7 or 8 days ($2\alpha \leq 0{\cdot}05$). There was no difference in rate of immobilisation of purified treponemes harvested after 7 or 8 days at any of the times indicated in fig 3. Pre-incubation of the 5-, 7- and 8-day treponemes with heat-inactivated NRS diminished their rate of immobilisation as compared with their counterparts which had been pre-incubated with BRM. The strongest effect was noted with 5-day treponemes. At all time points a significant difference was observed between 5-day treponemes on the one hand, and 7- and 8-day treponemes on the

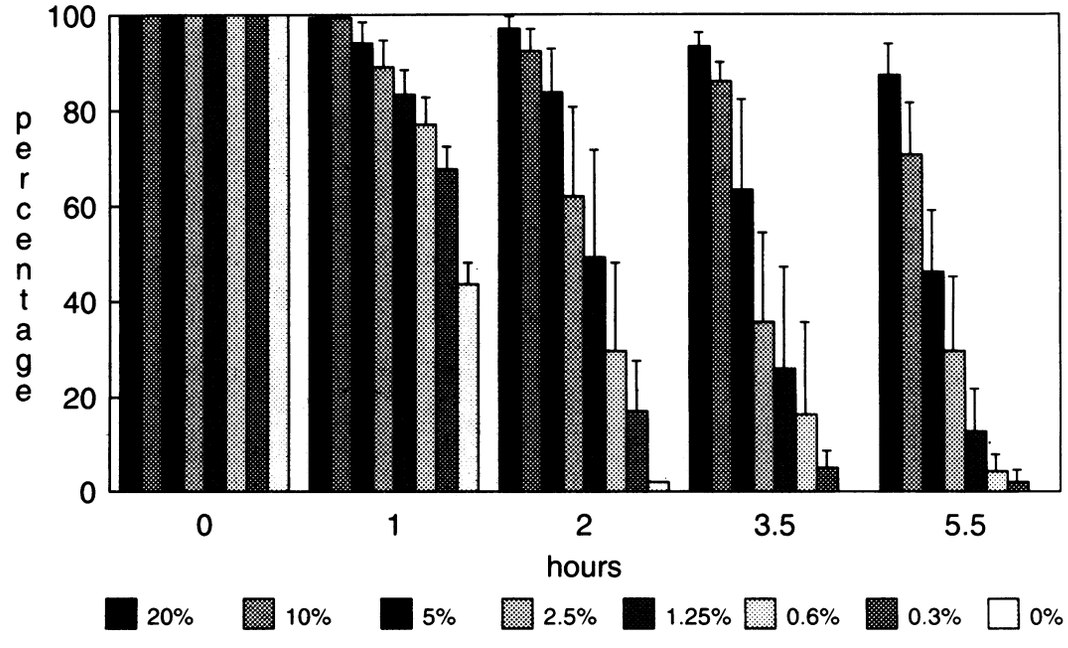

Figure 2 Treponemal mobility after preincubation with eight different percentages of NRS (20%–0%) are demonstrated. Results are expressed in percentage of mobile treponemes. Means and standard deviations of the results of experiments with three suspensions are shown.

Table 1 Immobilisation of Percoll purified Treponema pallidum after pre-incubation with sera from different species of animals. The mean percentages of mobile treponemes with the serum of one representative of each species, tested against three different treponeme suspensions are shown (range in parentheses)

	2 hours	*5·5 hours*
Pig	72·3 (55–89)	54·7 (34–71)
Hamster	5·7 (2–11)	1·0 (0–3)
Goat	18·0 (15–21)	4·3 (4–5)
Mouse	6·3 (3–10)	0·3 (0–1)
Dog	29·3 (19–44)	11·3 (5–17)
Rat	11·3 (2–25)	0·7 (0–1)
Guinea-pig	10·3 (8–18)	0·3 (0–1)
Human	2·0 (2–2)	0·0

other ($2\alpha \leq 0{\cdot}05$). No differences were seen between the 7- and 8-day treponemes (fig 3).

The effect of the level of anti-treponemal antibodies in the pre-incubation sera on immobilisation. Figure 4 shows that all sera used caused a delay in the immobilisation of the treponemes as compared with BRM. However, compared with the normal rabbit serum pool, the pre-incubations with the high-titred-, low-titred- and "autologous" serum resulted in a significantly more rapid immobilisation of the treponemes ($2\alpha \leq 0{\cdot}05$). The treponemes were immobilised significantly more slowly in low-

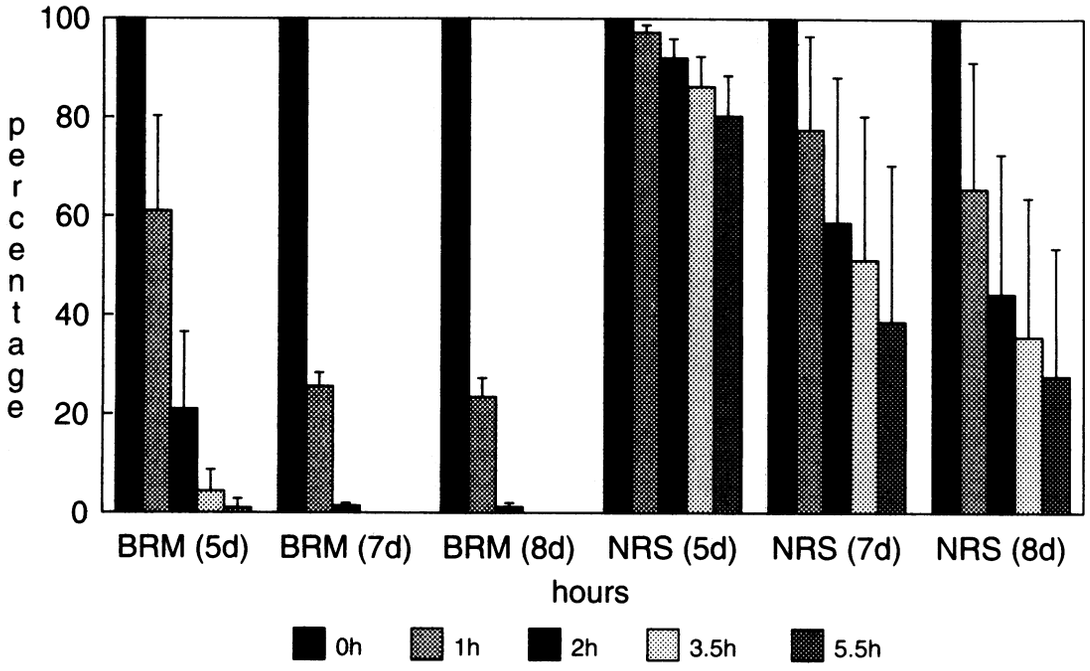

Figure 3 Immobilisation of Percoll purified treponemes using T pallidum harvested after different periods of infection (5, 7 or 8 days). Results are expressed as percentage of mobile treponemes. Means and standard deviations of the results of experiments with five suspensions obtained from different rabbits for each period of infection are shown.

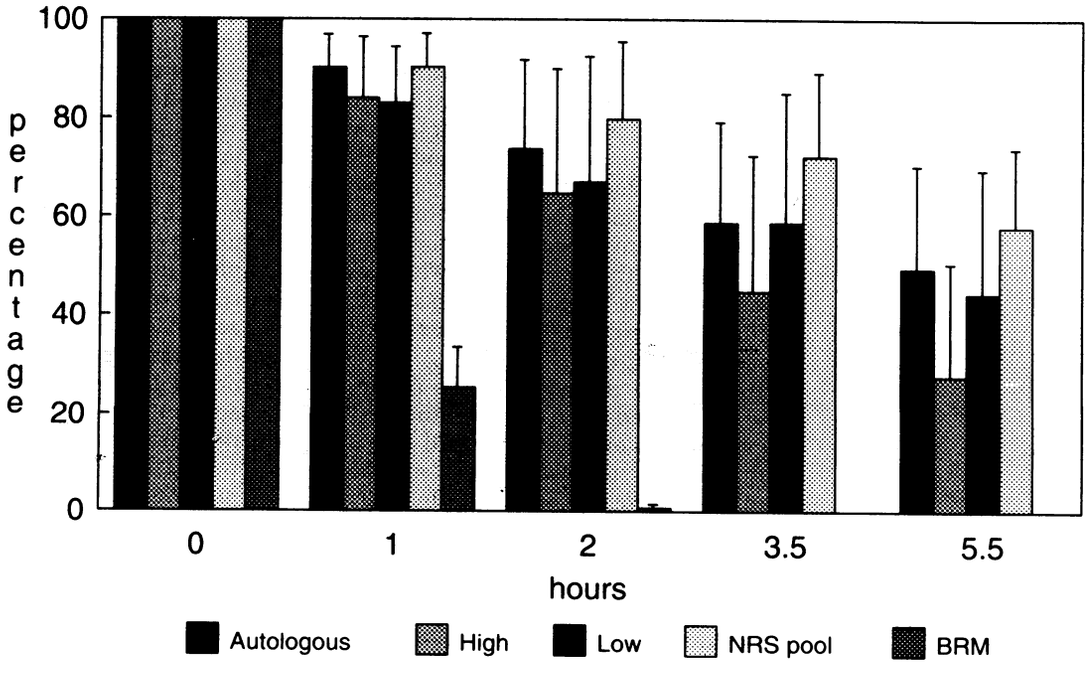

Figure 4 Percentage of mobile treponemes after preincubation with different heat-inactivated rabbit sera or BRM only, before addition of normal human serum as a complement source. Means and standard deviations of results of experiments with six treponeme suspensions isolated from different rabbits are shown.

Table 2 Mean percentage (range in parentheses) of mobile treponemes after two sequential preincubations with NRS followed by NRS; with NRS followed by BRM; and BRM followed by BRM, before the incubation with NHS, as determined with 5 different treponeme suspensions

	2 hours	5·5 hours
NRS/NRS	50·0 (25–81)	20·8 (7–34)
NRS/BRM	2·2 (0–9)	0
BRM/BRM	0·4 (0–2)	0

titred serum and in "autologous" serum than in high-titred serum ($2\alpha \leq 0{\cdot}05$).

Influence of removal of pre-incubation serum on immobilisation. In table 2 it is shown that the treponemes which were pre-incubated in BRM in both pre-incubation steps were immobilised after 2 h. The same result was noted for treponemes which were pre-incubated with NRS in the first and with BRM in the second pre-incubation step. However, after pre-incubation of the purified treponemes with heat-inactivated NRS in both pre-incubations a delay in the immobilisation was observed. After 2 h a mean mobility of 50% was noted.

Discussion

In a previous study we demonstrated that the rapid immobilisation of Percoll purified treponemes follows activation of complement along the classical pathway.[8] This suggests the participation of antibodies in the form of antigen-antibody complexes on the outer surface of the treponemes, which then initiates the classical complement cascade. The complement level of the human serum pool, measured as its haemolytic capacity by the CH_{50} method, was not changed by adding heat-inactivated NRS. Furthermore, the test mixtures used to study the immobilisation of the treponemes contained residual complement after 22 h of incubation, as was shown by their capacity to lyse sensitised sheep erythrocytes. As a consequence, neither an anti-complement effect nor a lack of complement was responsible for the delay in immobilisation of the treponemes pre-incubated in NRS, as compared with those pre-incubated in BRM or NHS. The inability to form antigen-antibody complexes may relate to a protective cover on the outer surface of the treponemes, preventing access of antibodies. The antibodies involved in immobilisation are derived from cross-reacting antibodies in the human serum pool used as the complement source, antibodies present on the surface of the treponemes, which were not removed by the purification procedure and/or antibodies present in the sera used in the pre-incubation steps.

In studying the possible role of antibodies that are carried over, we hypothesised that a shorter period of infection would lead to fewer antibodies on the treponemal surface, since Hanff *et al* have demonstrated that after infection of the rabbits, serum antibodies with anti-treponemal specificities increase from day 3 onwards.[14] Indeed, we noted that Percoll purified treponemes from 5-day infections were immobilised significantly more slowly than the purified treponemes from 7- and 8-day infections in otherwise identical reaction mixtures. This makes it likely that "carry over" antibodies may participate in the in vitro immobilisation of the purified treponemes and suggests that in the infected rabbit the newly formed antibodies may have access to the treponemal surface. This accords with the findings of Blanco *et al.*[15]

The study of the role of antibodies in the sera used for pre-incubation showed that pre-incubations with all three types of anti-treponemal antibody containing sera resulted in a significant delay in the immobilisation as compared with pre-incubations with BRM, but produced a significant acceleration of the immobilisation as compared with pre-incubations with the seronegative rabbit serum pool. This shows that anti-treponemal antibodies in the serum used for pre-incubation can participate in the immobilisation of the treponemes and demonstrates that the surface of the Percoll treponemes is accessible to antibodies, despite the presence of rabbit serum proteins. Taken together, these results indicate that it is unlikely that the rabbit sera provide a resistance to in vivo or in vitro immobilisation by formation of a physical barrier to antibodies.

Immunofluorescence revealed that irrespective of the sera used for pre-incubation, the treponemes were covered with a layer of human C3b after an hour of incubation with NHS. This shows firstly that the antibodies on the treponemal surface can act as initiators of the complement cascade despite the presence of rabbit serum proteins. Secondly this shows that a considerable part of the treponemes, pre-incubated in a serum able to provide resistance against rapid immobilisation, can survive in vitro for at least several hours despite the activation of complement on their surface. These findings are partly in agreement with those of a recent study in which it was demonstrated that antibody binding and complement-mediated immobilisation of unpurified treponemes were not the rate-limiting steps in the immobilisation of treponemes.[15] In that study the prolonged time required for in vitro immobilisation of the unpurified treponemes was related to the limited rate of complement activation. Aggregation of the treponemal rare outer membrane protein (TROMP) occurred in the presence of antibodies during in vitro incubation and was thought to be necessary to make binding of the first complement component by the antibodies possible.[15] The Percoll purified treponeme suspensions we used are relatively free of host proteins and rapidly activated complement, as was demonstrated by the presence of a C3b layer around the treponemes after an hour of incubation with NHS. It appears, therefore, that the purification procedure modifies the treponemal surface in such a way that it becomes capable of rapid binding of the first complement component, possibly by allowing a more rapid aggregation of the TROMP. Although this may explain the rapid activation of complement, it fails to explain the prolonged time needed for immobilisation of the purified treponemes after

a pre-incubation with sera, which delay the immobilisation.

After performing two sequential pre-incubations of the purified treponemes a decrease of the immobilisation rate was noted only in the test mixtures in which NRS had been present in both pre-incubations. The treponemes in those test mixtures in which BRM had been present in both pre-incubations or only in the second one were immobilised at the same (rapid) rate. This shows the necessity of presence of rabbit serum proteins in the reaction mixture to obtain a delay in the immobilisation and suggests that the inhibition of the immobilisation is caused by some mechanism that operates in the fluid phase. One possibility is that the rabbit serum proteins interact with the late-acting human complement components to prevent either their formation or their lytic effect. However, this seems unlikely, since sensitised sheep erythrocytes were lysed by the NHS/NRS mixtures. A second possibility is that an enzyme system needed for the immobilisation is competitively inhibited by the combination of human and rabbit serum. The participation of an enzyme in the immobilisation is not unprecedented. Müller *et al* have shown that in vitro immobilisation of unpurified treponemes does not occur in the complete absence of lysozyme.[16] It is not known whether additional enzymes play a role in the immobilisation of the treponemes. It might be possible that the dose dependent delay of immobilisation by NRS originates from an inhibitory effect on such (an) enzyme(s).

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